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## Regulation of osteoblast differentiation

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## Multiple mechanisms are involved in inhibition of osteoblast differentiation by PTHrP and PTH in KS483 cells

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## Abstract

We examined the mechanism by which PTHrP and PTH inhibit KS483 osteoblastic differentiation. We show that PTHrP and PTH inhibit differentiation downstream of early bone morphogenetic protein (BMP)-signaling and downregulated components of the hedgehog (Hh) signaling cascade. In addition, PTHrP and PTH repressed RunX2 and osterix (*osx*) expression. Overexpression of either gene, however, could not relieve PTHrP and PTH's inhibitory actions. Our data suggest that multiple parallel mechanisms are involved in the inhibition of osteoblast differentiation and matrix mineralization by PTHrP and PTH.

### Introduction

PTHrP and PTH are potent inhibitors of osteoblast differentiation *in vitro* by as yet unexplained mechanisms.

### Materials and Methods

We treated murine bone marrow stromal cells and the mesenchymal progenitor cell line KS483 with PTHrP and PTH in combination with either BMPs or Hh and measured early and late markers of osteoblast differentiation and studied the expression of RunX2 and *osx*. In addition, we examined PTHrP and PTH's response in stable KS483 cells overexpressing either RunX2 or *osx*.

### Results

PTHrP and PTH inhibit BMP- and Hh-induced osteogenesis downstream of early BMP-signaling and by downregulation of components of the Hh signaling cascade. PTHrP and PTH prevented the upregulation of RunX2 expression associated with osteoblast differentiation in an indirect response. However, PTHrP and PTH could still inhibit differentiation, and particularly matrix mineralization, of cells expressing RunX2. In addition, PTHrP and PTH potently downregulated *osx* expression only in mature osteoblasts in an intermediate early response, but *osx* overexpression could not relieve the inhibitory effects of PTHrP and PTH on matrix mineralization.

### Conclusion

Our data suggest that besides transcriptional repression of RunX2 and *osx* also other mechanisms in parallel with or downstream of RunX2 and *osx* are involved in the inhibition of osteoblast differentiation and matrix mineralization by PTHrP and PTH *in vitro*.

## Introduction

The critical role of parathyroid hormone (PTH) and its related peptide (PTHrP) in bone development is underscored by several natural and targeted knockouts of these genes and their receptor, the PTH1R, in human and mice. Loss of function of PTH1R in mice results in growth plate abnormalities due to premature, accelerated hypertrophic chondrocyte maturation<sup>(1)</sup>. Similar bone abnormalities are found in humans with Blomstrand lethal osteochondrodysplasia (BOCD), which is caused by an inactivating mutation in the PTH1R<sup>(2)</sup>. The converse findings, i.e. skeletal abnormalities due to decelerated chondrocyte maturation, caused by an activating mutation in the PTH1R, are observed in humans with Jansen's type metaphyseal chondrodysplasia (JMC)<sup>(3)</sup>. Introducing the same mutation in chondrocytes of transgenic animals also delayed hypertrophic chondrocyte differentiation and matrix mineralization<sup>(4)</sup>. Based on experiments in transgenic animals, it was shown that the ligand mediating these effects on endochondral bone formation is PTHrP<sup>(5,6,7,8)</sup>. PTH does play only a minor role in endochondral bone development, since PTH deficient pups are viable and display only mild defects in the growth plate. In addition, these newborns show a reduced amount of osteoblasts and trabecular bone<sup>(9)</sup>.

Although the most striking abnormalities of PTH1R and PTHrP knockout and transgenic animals were found in the growth plate, they also displayed changes in osteoblast differentiation. PTH1R null mice showed decreased trabecular bone, an increase in cortical bone and abnormal bone mineralization<sup>(10)</sup>. In addition, increased trabecular and cortical bone mass was found in patients with BOCD<sup>(11)</sup>. Loss of cortical bone and augmentation of trabecular bone is seen in patients with JMC<sup>(12)</sup>. Specific targeting of a constitutively active PTH1R to osteoblasts in mice led to increased osteoblast differentiation in trabecular bone and at the endosteal surface of cortical bone, whereas in the periosteum osteoblastic differentiation was decreased<sup>(13)</sup>. Finally, mice heterozygous for PTHrP deletion develop osteopenia when reaching adulthood, which is in part due to decreased osteoblast formation (reviewed in<sup>(14)</sup><sup>(15)</sup>). Remarkably, at 4 months of age PTH-deficient mice have increased trabecular bone volume which can be attributed to increased expression of PTHrP by osteoblasts<sup>(16)</sup>. Summarizing, the PTH1R and PTHrP play an important role in osteoblastic bone development and maintenance of trabecular bone volume, which is a relevant finding with respect to the anabolic effect of PTH and PTHrP on bone when administered to patients with osteoporosis<sup>(17,18)</sup>.

However, despite all this, relatively little is known about the role of PTH1R signaling in osteoblast differentiation. Numerous reports have addressed the role of PTHrP and PTH during osteoblast differentiation *in vitro*. Several groups showed that PTHrP increased the proliferation of osteoprogenitor cells and osteoblasts *in vitro* and *in vivo*<sup>(19,20,21)</sup>, and decreased apoptosis of pre-osteoblasts and osteoblasts, thereby increasing the amount of osteoblasts<sup>(22,23)</sup>. In addition, in the majority of these studies, PTHrP and PTH inhibited osteoblast differentiation as measured by alkaline phosphatase activity and nodule formation using various models, such as UMR 106, calvarial primary osteoblastic cells and human osteoblast-like cells<sup>(19,24,25)</sup>. Few studies showed increased osteoblast differentiation<sup>(26,27)</sup>, and current data suggest that these effects of PTHrP or PTH are cell type, differentiation stage, dosage and exposure time dependent<sup>(27,28,29,30)</sup>. The reason for this discrepancy is currently unknown.



Signaling events downstream the PTH1R have been extensively investigated, and involve activation of multiple G proteins, namely G $\alpha_q$ , with subsequent activation of phospholipase C (PLC) and G $\alpha_s$  which activates adenylate cyclase, respectively<sup>(31)</sup>. The mechanism by which PTHrP exerts its effects on osteoblastic bone development is largely unknown. Since PTHrP itself does not possess the capacity to induce osteoblast differentiation from committed precursor cells, PTHrP might influence the activity of the bone forming capacity of members of the Bone Morphogenic Protein (BMP), or Hedgehog (Hh) family of morphogens, which have been shown to play an important role in the initiation and progression of osteoblast differentiation<sup>(32,33,34,35)</sup>.

Furthermore, PTHrP and PTH might influence either the expression or post-translational modifications of the bone-specific transcription factors RunX2 and osterix (osx). Both transcription factors are downstream targets of BMPs and Hedgehogs and lack of these factors impairs osteoblast differentiation<sup>(36,37,38,39,40,41)</sup>.

To address these issues, we studied molecular mechanisms by which PTHrP and PTH control osteoblast differentiation by interference with the above-described osteogenic pathways at various levels. For these studies, mainly the murine mesenchymal progenitor-like KS483 cell line has been used for *in vitro* differentiation assays. In these cells, both PTHrP and PTH dose-dependently decrease osteoblastic differentiation independently upon administration time or duration of treatment. PTHrP and PTH also inhibited BMP- and Hh-induced osteoblast differentiation and matrix mineralization. Furthermore, PTHrP and PTH-treatment prevented the increase in RunX2 expression associated with proceeding of osteoblast differentiation. However, forced overexpression of RunX2 and one of its downstream targets, osx, was not able to reverse the inhibitory effects of PTHrP and PTH. Our data indicate that PTHrP and PTH use various mechanisms to inhibit osteoblast differentiation and matrix mineralization in KS483 cells.

## Materials and methods

### Cell culture

KS483 cells were cultured routinely as described previously<sup>(32,34)</sup>. Primary bone marrow cells were derived from tibia and femur of 7 week-old male mice (FVB) and cultured in  $\alpha$ -MEM (GIBCO BRL) supplemented with penicillin/streptomycin, 50  $\mu$ g/ml ascorbic acid and 10% heat inactivated FCS. Stable cell lines were generated using the Flp-In system (Invitrogen) according to the manufacturer's protocol. In short, first a KS483 Flp-in host cell line was generated by stable introduction of a single copy of an Flp-Recombinase target (FRT) site as an integral part of an antibiotic resistancy gene in the genome of KS483. One clone, KSfrt 3B2, was selected that was comparable to the parental KS483 cells in differentiation characteristics and retained full capacity to differentiate into osteoblasts. This clone was used for the generation of the isogenic stable cell lines by transient co-transfection of an FRT-targeting vector and an Flp-recombinase expression vector. The FRT-targeting vector was either an empty pEF5/FRT/V5-DEST vector (Invitrogen) as control or a pEF5/FRT/V5-DEST vector expressing RunX2 or osterix under the control of the EF1 $\alpha$  promoter. Due to Flp-mediated recombination at the genomic FRT-site, this targeting vector was incorporated in the genome. Simultaneously, a shift in antibiotic resistancy was introduced allowing positive selection for integrants

in the genomic FRT-site only and negative selection for random integrants in one single step. This method allows the generation of an isogenic stable cell lines, which only differ in the sequence inserted in the genomic FRT site, thereby eliminating the need for clonal selection and analysis of multiple clones with variable expression of the transgene due to uncontrolled integration of the transgene in the genome.

### Differentiation studies

To study effects on ALP activity, KS483 cells were cultured until confluence and thereafter cultured for 3 days with ascorbic acid in the absence or presence of recombinant human BMP-4 (R&D systems, Uithoorn, The Netherlands), recombinant human BMP-6 (R&D systems), recombinant depalmitoylated human sonic hedgehog (kindly provided by Dr. A. Esswein, Boehringer Mannheim, Germany), parathyroid hormone (PTH) (1-34), (3-34) or (7-34) (range of  $10^{-7}$  to  $10^{-11}$  M, Bachem Holding AG, Bubendorf, Switzerland), parathyroid hormone related peptide (PTHrP) (1-34) (range of  $10^{-7}$  to  $10^{-11}$  M, Bachem Holding AG). At day 7 or 11, the supernatants were withdrawn and the cells were washed twice with PBS. ALP activity and DNA content were extracted from the cell layer and measured as described previously<sup>(65)</sup>. Values represent the average  $\pm$  SEM of 2 independent triplicate experiments corrected for DNA content and are expressed as mOD units/ minute or as percentage of control.

KS483 long-term differentiation studies were performed as described previously<sup>(32)</sup>. Bone marrow cells were seeded at a density of 500.000 cells / cm<sup>2</sup>. In the first eleven days, 2/3 and thereafter the whole amount of medium was changed every 3 to 4 days. When nodules appeared (from day 11 of culture onwards)  $\beta$ -glycerolphosphate (5 mM, Sigma Chemical Co., St Louis, MO) was added to the culture medium. After three weeks of culture, cells were washed with phosphate buffered saline (PBS), and fixed with 3.7% buffered formaldehyde for 10 minutes. The cultures were analyzed for osteogenesis and DNA content as described previously<sup>(34)</sup>. Values represent the average  $\pm$  SEM of 3 independent duplicate experiments corrected for DNA content and are expressed as percentage of control. Pictures of the images were taken and digital imaging was performed (representative images are shown).

### Transfection and assay for luciferase activity

KS483 cells were seeded at a density of 9,500/cm<sup>2</sup> in 24-wells plates, and transiently transfected as described previously with the BMP reporter constructs, Msx2-luc, SBE-luc and (BRE)<sub>4</sub>-luc<sup>(34)</sup>. To correct for transfection efficiency, 100 ng of renilla luciferase (pRL-SV40; Promega, Madison, USA) was co-transfected. 12 hrs after transfection, the medium was changed for medium containing 0.2% FCS. The cells were kept in 0.2% FCS medium for 1 day, and then left either non-stimulated or stimulated for an additional 24 hrs. Luciferase assays were performed with the Dual-Luciferase Reporter assay system (Promega) according to the protocol. 10  $\mu$ l of cell lysate was first assayed for firefly luciferase and then for Renilla luciferase activity, using the Wallac 1450 Microbeta Trilux luminescence counter (Perkin-Elmer, Boston, USA). Firefly luciferase activity was corrected for renilla luciferase activity. Values represent the average  $\pm$  SEM of 3 independent triplicate experiments corrected for transfection efficiency and are expressed as fold induction.



## cAMP measurements

KS438 cells were cultured and subsequently challenged with hPTHrP (1-34). After 10 minutes, generation of intracellular cAMP was measured as described previously<sup>(42)</sup>.

## RT-PCR and real time PCR

RNA isolation was performed by the method described by Chomzynski and Sachi<sup>(43)</sup> and reverse transcribed into cDNA. cDNA was standardized by competitive PCR using an internal standard (pMUS) as described by van Bezooijen et al<sup>(44)</sup>. Subsequently, the expression of various genes was examined by semi-quantitative PCR as described previously<sup>(32)</sup>.

For real time PCR, the Eurogentec Kit for SYBR Green I has been used according to the manufacturer's protocol. Subsequently, the relative levels of cDNA were determined using the comparative method of Livak and Schmittgen<sup>(45)</sup>. Data were corrected for  $\beta$ 2 microglobulin ( $\beta$ 2M) expression. For used primersets see table 1. In short, of each time point, the Ct of  $\beta$ 2M and the gene of interest were determined for both control and PTH or PTHrP stimulated samples. Subsequently, values were corrected for  $\beta$ 2M expression relative to control / time point or relative to the control clone at day 4. Fold change was calculated according to the formula  $2^{-\Delta\Delta Ct}$ . For visualization, this value was log transformed. Data are representative for three separate experiments.

## Western Blot

For Western Blot, whole cell lysate was isolated by scraping the cells in PBS. The cell pellet was collected and lysed in 200  $\mu$ l lysisbuffer (20mM Tris pH 7,5; 20% glycerol; 400 mM KCl; 1 mM DTT; aprotinin (1/1000) and Boehringer protease inhibitor mix) followed by three subsequent rounds of freeze / thawing. The protein concentration was determined and 10  $\mu$ g protein was used for a Western Blot as described earlier<sup>(16)</sup>. As primary antibody, the rabbit polyclonal  $\alpha$ -PEBP2 $\alpha$ A (M-70) was used (Santa Cruz Biotechnology, Inc, USA). As a secondary antibody, the  $\alpha$ -rabbit-HRP was used, followed by staining with SuperSignal West Pico chemiluminescent substrate (Pierce).

## Statistics

Values represent mean  $\pm$  SEM. Differences were examined by multivariate analysis of variance (ANOVA) followed by the post-hoc least significant difference test (LSD). Results were considered significant at  $p < 0.05$ .

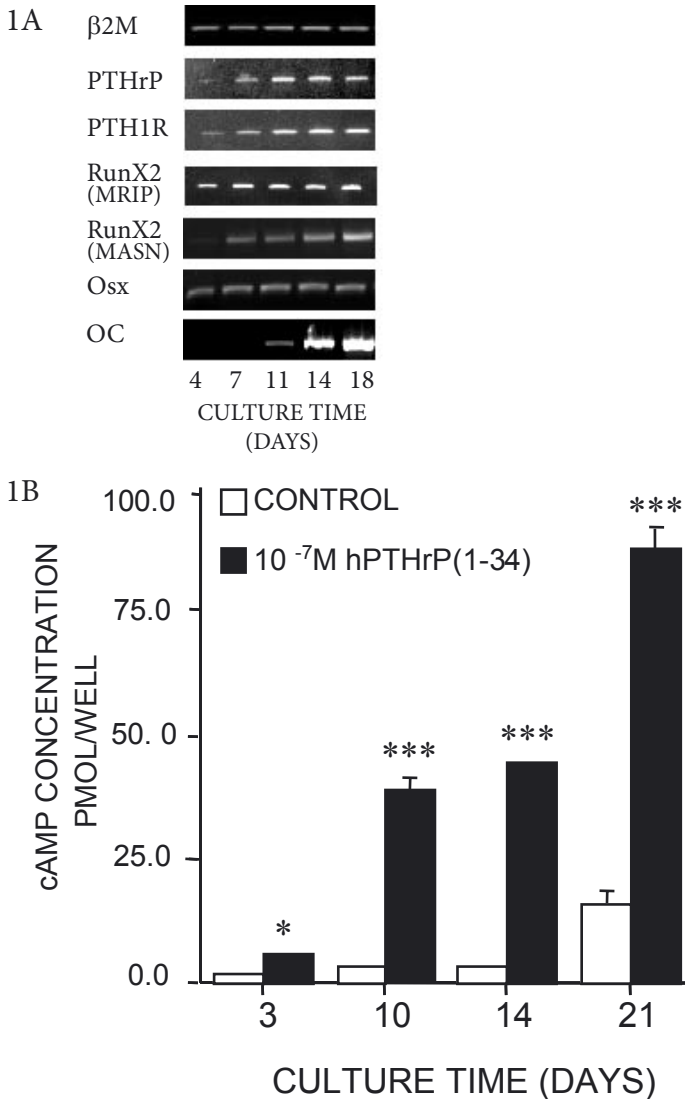
# Results

## Parathyroid hormone and its related peptide (PTHrP) inhibit osteoblastic differentiation of murine bone marrow MSC and KS483 cells

To study a putative autocrine role of PTHrP signaling in osteoblastic differentiation of mesenchymal progenitor-like KS483 cells, we first examined mRNA expression of PTHrP and the PTH1R (fig. 1A). Expression of PTHrP mRNA increased during KS483



osteoblast differentiation, in parallel with the increased expression of the osteoblast-specific splice variant of RunX2 (MASN), while the expression of the MRIP-splice variant did not change. Remarkably, mRNA expression of the transcription factor osterix (osx) did not change during KS483 osteoblastic differentiation (fig. 1A). In agreement with the increased mRNA expression of the PTH1R, responsiveness to PTHrP increased with differentiation as determined by measuring intracellular cAMP accumulation after a challenge with hPTHrP (1-34)(fig. 1B).

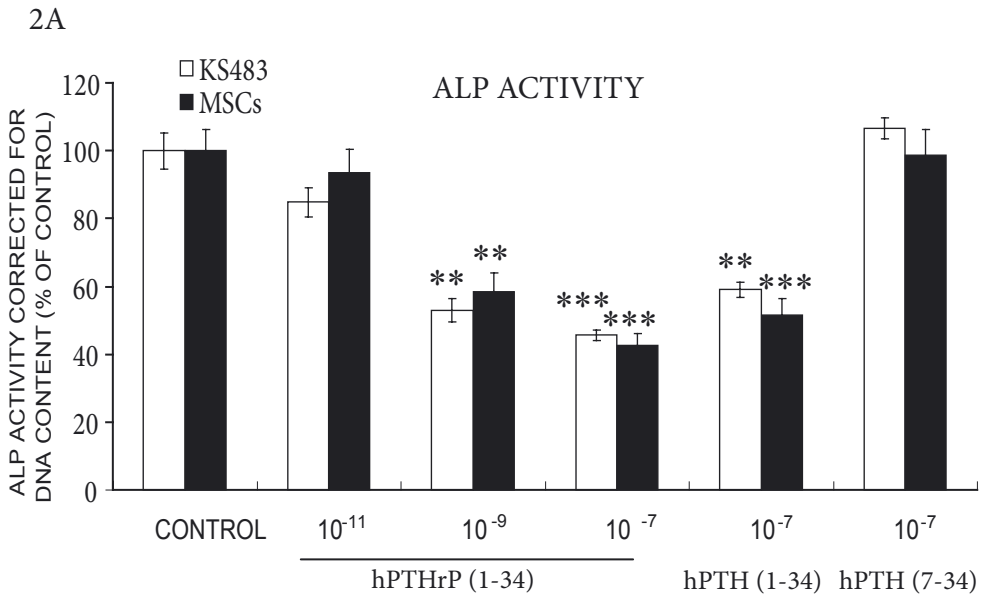


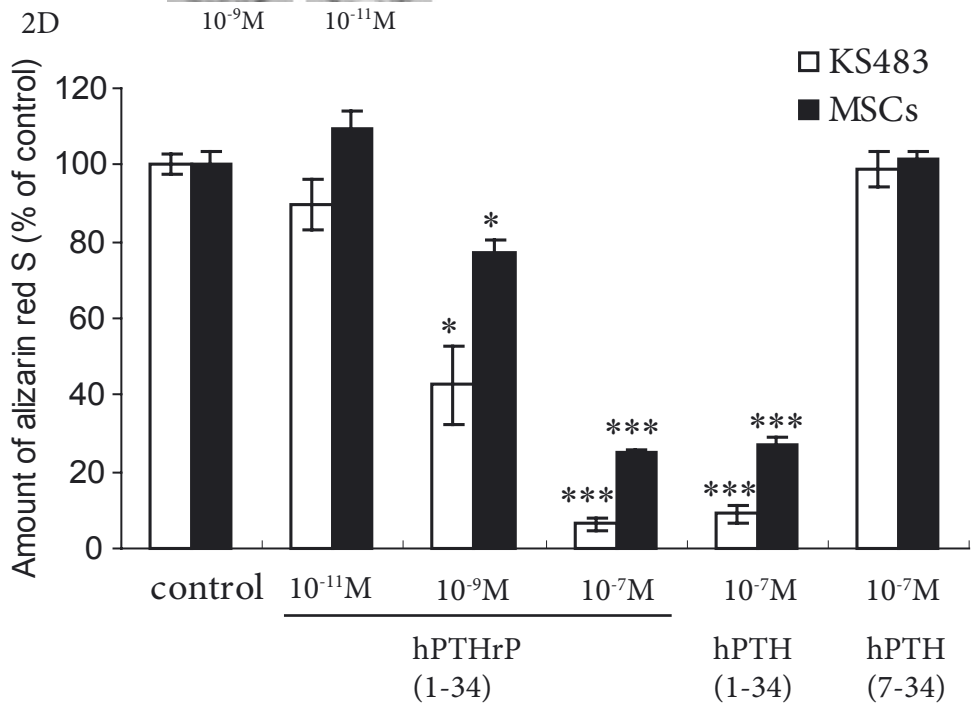
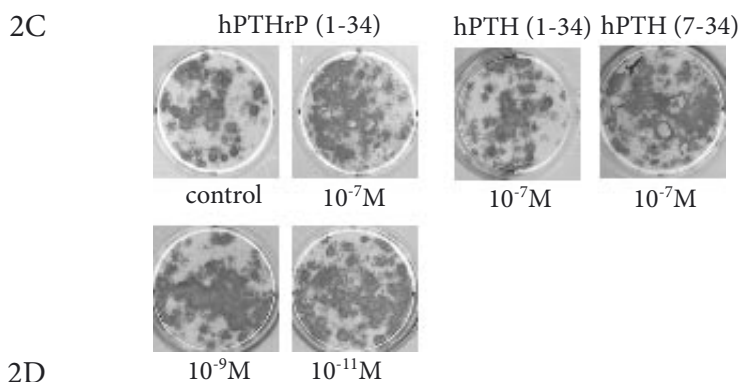
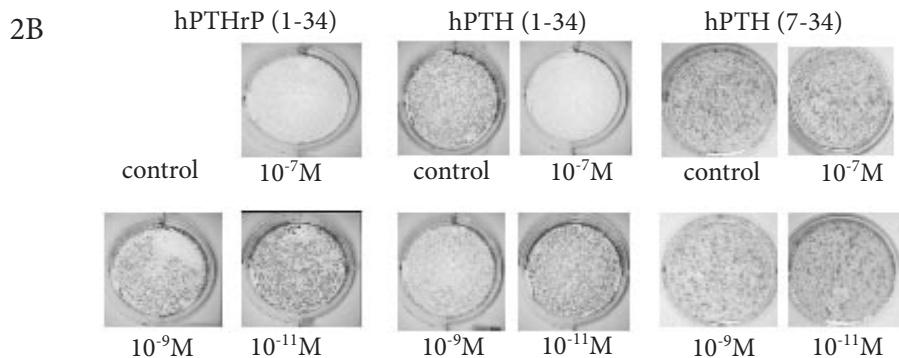
**Fig. 1 Autocrine role of PTHrP in KS483 osteoblastic differentiation**

(A) Expression of the indicated genes at day 4, 7, 11, 14 and 18 of KS483 osteoblastic differentiation. (B) Responsiveness to  $10^{-7}$ M hPTHrP (1-34) increased with differentiation as determined by measuring cAMP \*  $p < 0.05$  and \*\*\*  $p < 0.001$  vs control.



Subsequently, effects of exogenously added PTHrP or PTH on osteoblast differentiation of MSCs and KS483 cells were investigated. In short-term experiments, hPTHrP (1-34) as well as hPTH (1-34) inhibited the induction of the early osteoblast marker Alkaline Phosphatase (ALP) dose-dependently in both cell types (fig. 2A). In contrast, hPTH (7-34), which lacks the capacity to activate the intracellular cAMP pathway, had no effect. Neither PTHrP nor PTH fragments had an effect on cell proliferation as measured with a MTS assay (data not shown). Subsequently, the effects of PTHrP and PTH on bone nodule formation were determined. KS483 and MSC cells were cultured for respectively 18 and 21 days in the presence of hPTHrP (1-34), hPTH (1-34) or hPTH (7-34) and stained for ALP and amount of mineral with Alizarin Red S. Continuous addition of hPTHrP (1-34) or hPTH (1-34) significantly decreased the amount of ALP positive nodules dose-dependently in KS483, but not in MSC cells (representative images shown in respectively fig. 2B and 2C). In contrast, continuous addition of hPTHrP (1-34) or hPTH (1-34) significantly decreased nodule formation and matrix mineralization in both cell types, dose-dependently (fig. 2D). These effects were also dependent upon cAMP signaling, since the 7-34 analogue did not affect the amount of ALP-positive and mineralized nodules. These data were corroborated by expression data demonstrating that continuous treatment of KS483 cells with hPTHrP (1-34) decreased the expression of established osteoblast markers, such as osteocalcin, PTH1R and ALP (data not shown).







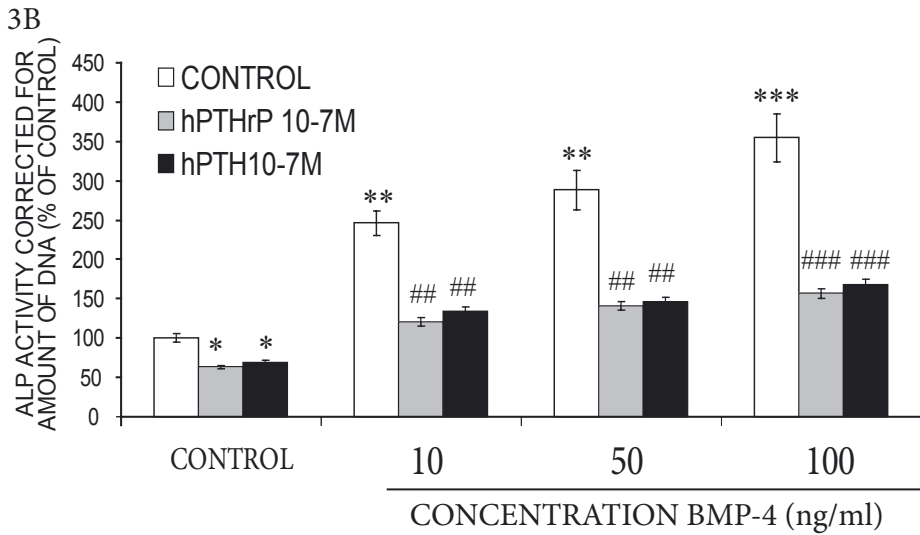
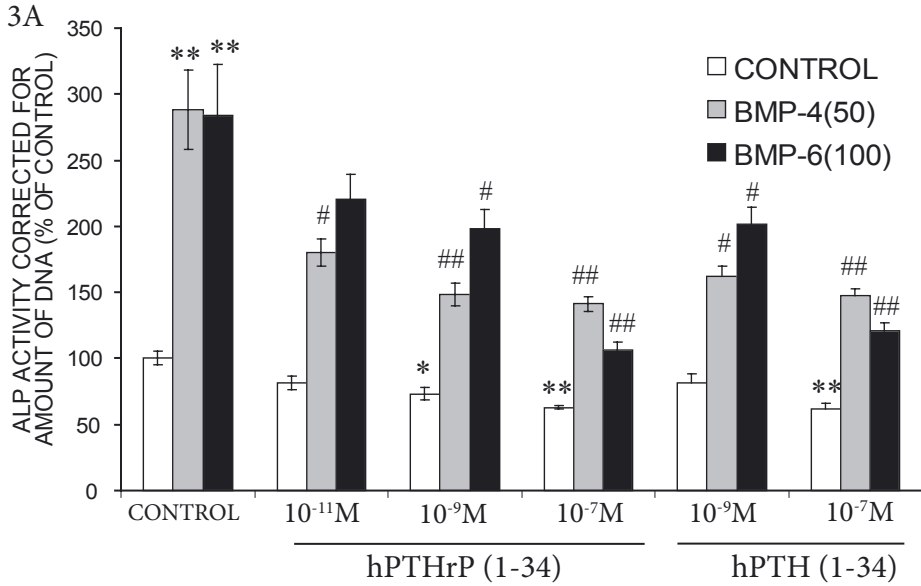
**Fig. 2 PTHrP and PTH inhibit osteoblast differentiation of MSCs and KS483 cells.**

Treatment of KS483 and MSCs cells with hPTHrP (1-34) or hPTH (1-34) resulted in a dose-dependent decrease in ALP activity at day 11 of culture. No effects were found after treatment with hPTH (7-34) (A). Continuous treatment of KS483 cells with hPTHrP (1-34) or hPTH (1-34) resulted in a dose-dependent decrease in amount of ALP positive nodules at day 18 of culture. No effects were found using hPTH (7-34) (B). Continuous treatment of MSCs cells with a dose-range of hPTHrP (1-34) or 10<sup>-7</sup> M hPTH (1-34) or hPTH (7-34) had no significant effect on the amount of ALP positive nodules after 21 days of culture (C). The amount of mineral was dose-dependently decreased in both KS483 and MSCs cells after treatment with hPTHrP (1-34) or hPTH (1-34). No differences were seen in the hPTH (7-34) treated cultures (D). \* p<0.05, \*\* p<0.01, \*\*\*p<0.001 vs control.

It has been shown previously that the way of administration of PTHrP or PTH, either continuously or pulsatile, may have opposite effects on osteoblast differentiation<sup>(47,48,27,30)</sup>. To study this, we treated KS483 and MSCs cells for respectively 18 and 21 days with hPTHrP (1-34) either continuously or in 1hr pulses at 3 to 4 days intervals. However, both treatments inhibited differentiation with comparable efficiency (data not shown). Furthermore, inhibitory effects on KS483 osteoblastic differentiation were also found in time window experiments in which hPTHrP (1-34) or hPTH (1-34) was added during the first or second week of osteoblast differentiation. For example, treatment of late-stage mineralizing cultures in the second week of culture with a high dose of hPTHrP (1-34) or hPTH (1-34) caused a rapid decrease in the number and size of ALP positive and mineralized nodules (data not shown).

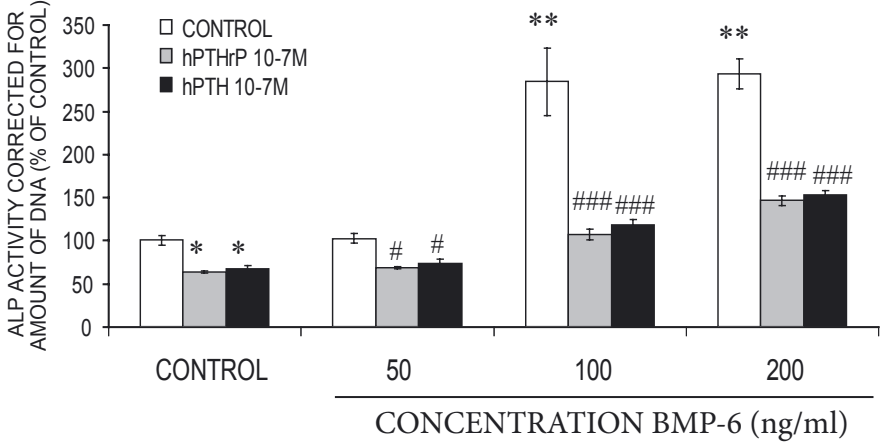
### Cross talk between Bone Morphogenetic Proteins (BMPs) and PTH/PTHrP signaling

Previously, we reported that autocrine BMP signaling is crucial for osteoblastic differentiation of KS483 cells<sup>(32)</sup>. It is feasible that PTHrP and PTH inhibit osteoblast differentiation by counteracting BMP signaling. Therefore, KS483 cells were cultured until confluence and thereafter treated for an additional 72 hrs with either BMP-4, -6, hPTHrP (1-34), or hPTH (1-34), and combinations of these. hPTHrP (1-34) or hPTH (1-34) dose-dependently inhibited both BMP-4 and BMP-6 induced ALP activity (fig. 3A). BMP-4 and -6 could only partly overcome the inhibition induced by hPTHrP (1-34) or hPTH (1-34) (respectively fig. 3B and 3C). In long-term cultures (18 days), PTHrP or PTH inhibited both basal and BMP-4 induced ALP activity (data not shown), the number of nodules, as well as the amount of deposited mineral dose-dependently (fig. 3D). To determine whether early BMP signaling was involved in the PTHrP or PTH-induced inhibition of differentiation, KS483 cells were transiently transfected with the Smad dependent promoter-reporter constructs BRE, Msx2 or SBE coupled to luciferase<sup>(49)</sup>. Neither hPTHrP (1-34) nor hPTH (1-34) affected basal, BMP-4 or BMP-6-induced luciferase expression of the BMP reporter BRE-luc (fig. 3E; BMP-6 data not shown). Similar data were obtained for the BMP reporters Msx2-luc and SBE-luc (data not shown). In contrast, dominant negative forms of the receptor-mediated Smads involved in the BMP pathway, Smad1 and Smad5, significantly decreased both basal and BMP-induced luciferase activity. In addition, dominant negative p38 as well as the p38 MAPK inhibitor SB203580 inhibited luciferase activity, in line with its role in BMP signaling<sup>(50,51,52)</sup>.

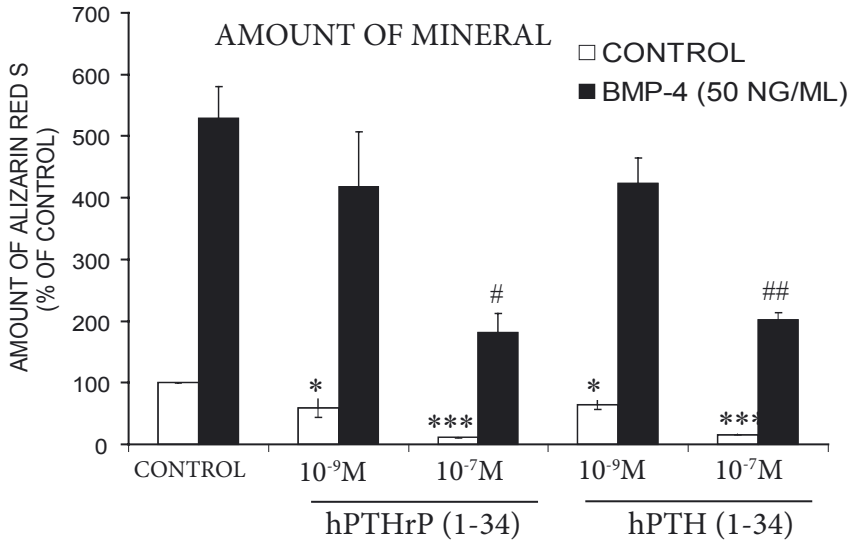




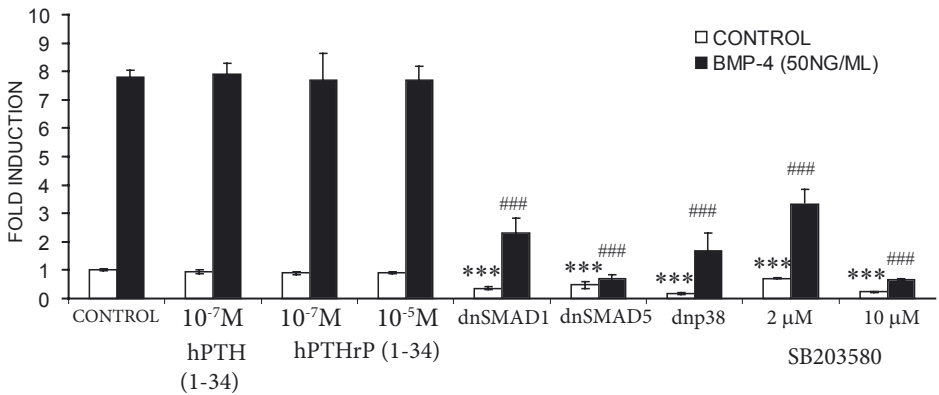
3C



3D



3E



**Fig. 3 Interactions between PTH/PTHrP and the BMP signaling pathway.**

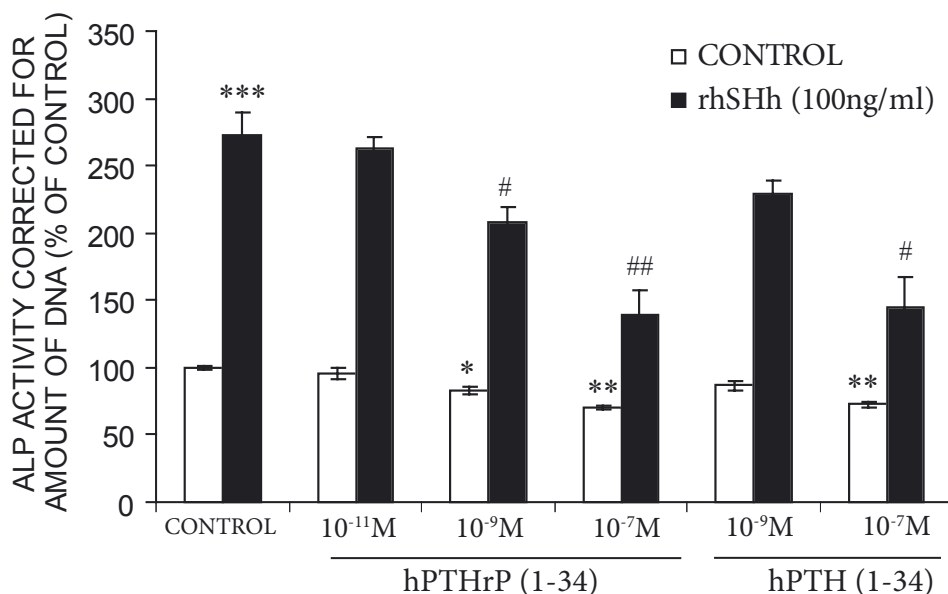
KS483 cells were cultured until confluence (day 4) and thereafter treated for 72 hrs. Treatment with hPTHrP (1-34) or hPTH (1-34) dose-dependently decreased both basal and BMP-4 (50 ng/ml) or BMP-6 (100 ng/ml)-induced ALP activity in KS483 cells (A). Treatment with BMP-4 (B) and -6 (C) could only partly overcome the inhibition of ALP activity induced by hPTHrP (1-34) or hPTH (1-34). Continuous treatment (18 days) with hPTHrP (1-34) or hPTH (1-34) resulted in a dose-dependent inhibition of the amount of deposited mineral in both control and BMP-4 treated cultures (D). Dominant negative constructs of Smad1, Smad5 or p38 MAPK, and treatment with p38 MAPK inhibitor SB203580 (2 or 10  $\mu$ M) decreased both basal and BMP-4 induced BRE-luciferase activity. Treatment with hPTHrP (1-34) or hPTH (1-34) for 24 hrs had no effect on the basal or BMP-4 induced activity of the BMP reporter BRE-luciferase. \* significant vs control; # significant vs BMP-treated control.

### Crosstalk between Hedgehog and PTH/PTHrP signaling

PTHrP or PTH might affect osteoblast differentiation by interfering with another important pathway, the Hedgehog pathway<sup>(34)</sup>. As shown in figure 4A, rShh-induced ALP activity was inhibited by both hPTHrP (1-34) and hPTH (1-34) dose-dependently. In addition, rShh could only partly overcome both hPTHrP- and hPTH-mediated inhibition of ALP activity (fig. 4B).

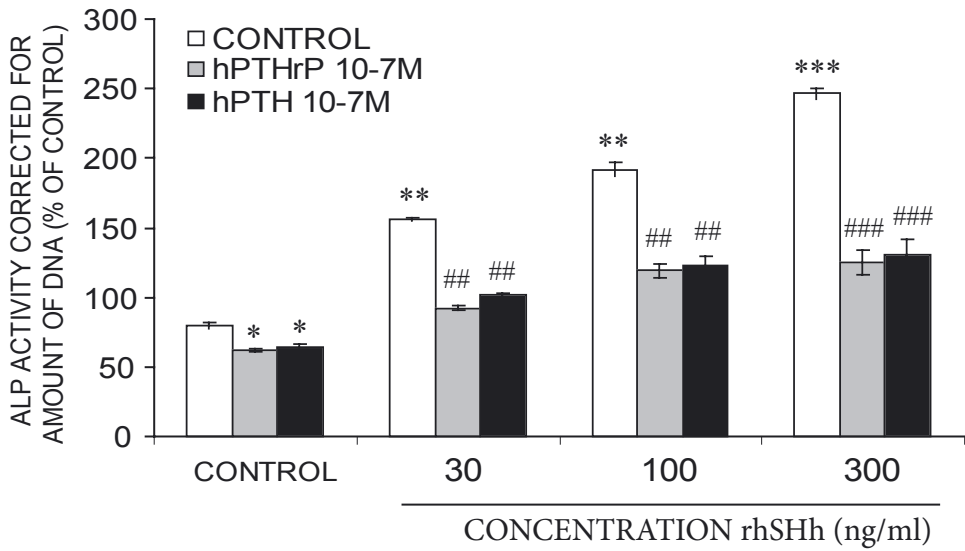
In long-term experiments, hPTHrP (1-34) or hPTH (1-34) dose-dependently inhibited the formation of ALP positive (data not shown) and mineralized nodules (fig. 4C). Furthermore, treatment of undifferentiated KS483 cells for 72 hrs with hPTHrP (1-34) decreased mRNA expression of Indian Hedgehog (Ihh) in KS483 cells (fig. 4D). In addition, mRNA expression of the Hh receptor patched 1 (Ptc1) as well as the intracellular signaling molecules Gli1 and Gli3, but not Gli2 was downregulated (fig. 4D).

4A

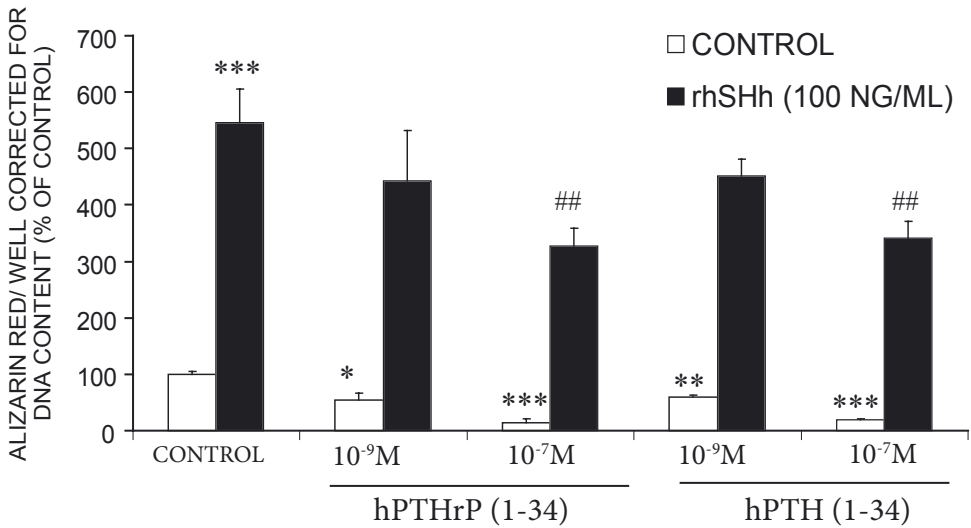




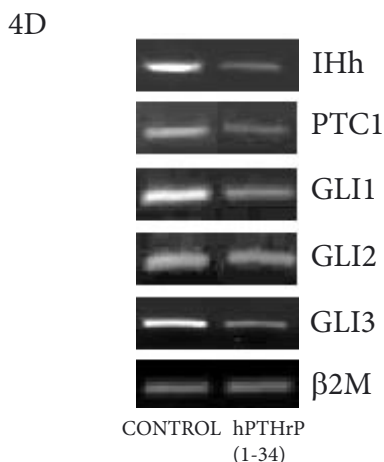
4B



4C







**Fig. 4 Interactions between PTH/PTHrP and Hedgehog signaling.**

KS483 cells were cultured until confluence (day 4) and thereafter treated for 72 hrs. Treatment with hPTHrP (1-34) or hPTH (1-34) dose-dependently decreased both basal and rhSHh-induced ALP activity in KS483 cells (A). Treatment with rhSHh could only partly overcome the inhibition of ALP activity induced by hPTHrP (1-34) or hPTH (1-34) (B). Continuous treatment (18 days) with hPTHrP (1-34) or hPTH (1-34) resulted in a dose-dependent inhibition of the amount of deposited mineral in both control and rhSHh treated cultures (C). mRNA expression of IHh, Ptc1 and its intracellular signaling molecules Gli1, Gli3 was decreased in undifferentiated KS483 cells (day 4) treated for 72 hrs with  $10^{-7}$  M hPTHrP (1-34). The expression of Gli2 and β2 microglobulin did not change (D). \* significant vs control, # significant vs rhSHh treated control

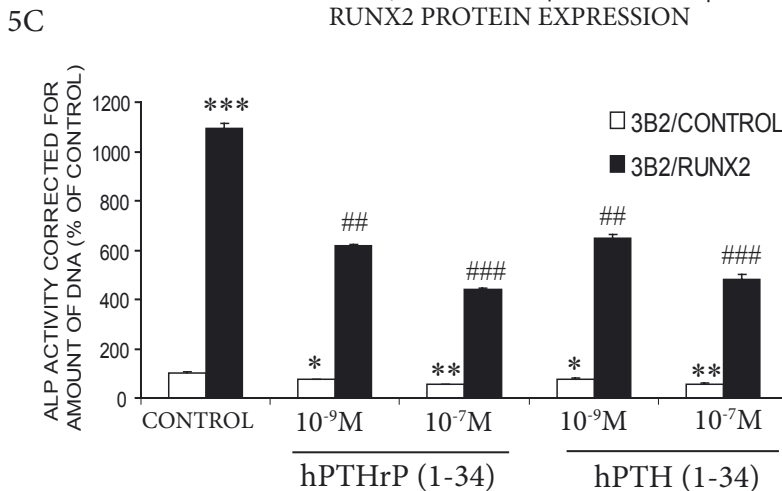
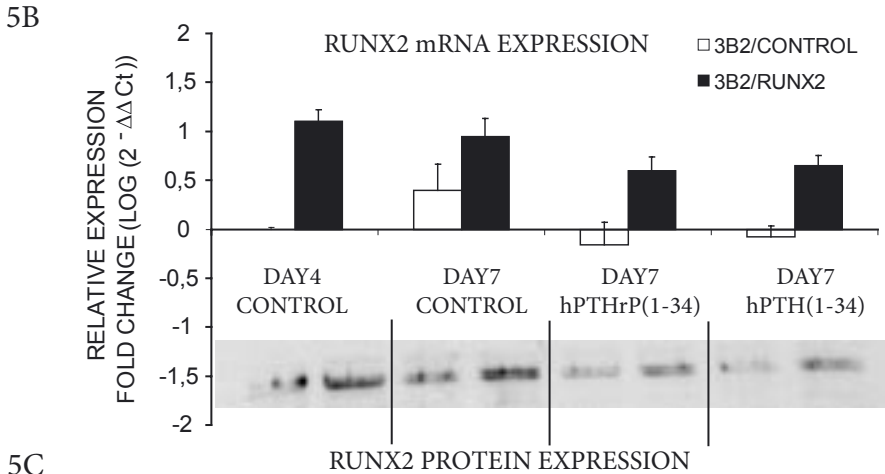
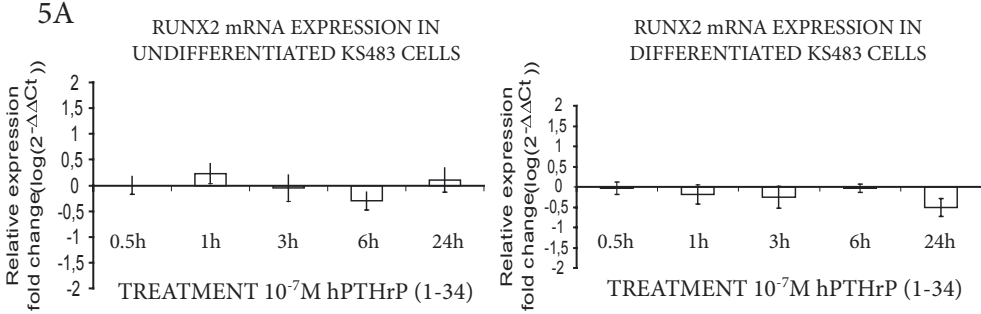
## Crosstalk between RunX2 and PTH/PTHrP signaling

The MASN-splice variant of the transcription factor RunX2 is indispensable for osteoblast differentiation and is a downstream target of BMP and Hh induced osteogenesis.

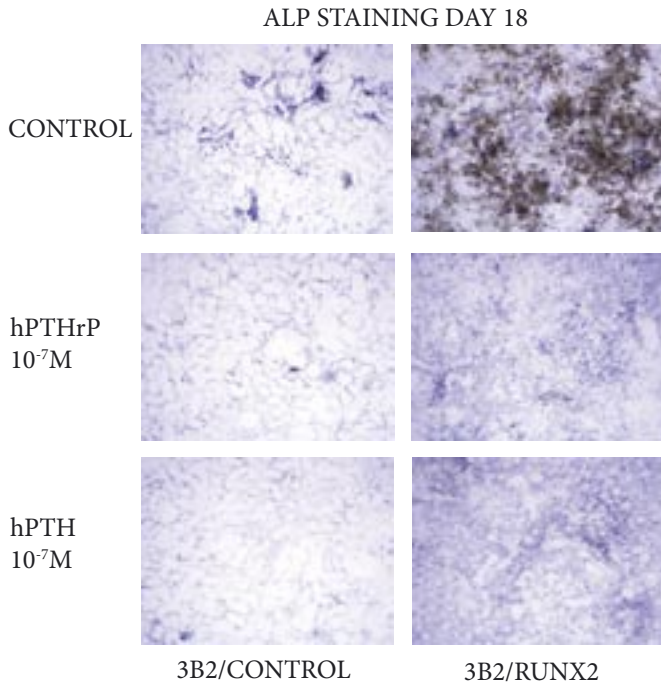
To test whether PTHrP interferes with RunX2 transcription, we studied its mRNA expression in undifferentiated (day 4) and differentiated (day 11) KS483 cells after treatment with hPTHrP (1-34) for respectively 30 minutes, 60 minutes, 3h, 6h or 24h. As shown in figure 5A, treatment with hPTHrP (1-34) had no effect on mRNA expression of RunX2 in both undifferentiated and differentiated KS483 cells. In contrast, treatment of undifferentiated KS483 cells with hPTHrP (1-34) for 72 hours prevented the increase in RunX2 mRNA and protein expression normally observed during osteoblast differentiation (fig. 5B). Subsequently, we tested whether forced overexpression of RunX2 could reverse the inhibitory effects of PTH and PTHrP on osteoblast differentiation. To test this, we used a subclone of the KS483 cell line, KSFrt 3B2/RunX2, overexpressing the MASN-splice variant of RunX2 at the mRNA and protein level (fig. 5B and chapter 4). Treatment of this clone with PTHrP or PTH for 72 hrs modestly reduced mRNA expression of RunX2, but levels remained higher than in the control clone. The downregulation paralleled a comparable modest decrease in RunX2 protein expression (fig 5B). Overexpression of RunX2 resulted in a dramatic increase in ALP activity (fig. 5C) and formation of ALP positive and mineralized nodules (fig. 5D and E). In short-term experiments, both hPTHrP (1-34) and hPTH (1-34) dose-dependently inhibited the RunX2 induced increase in ALP activity (fig. 5C) (at  $10^{-7}$ M PTHrP 46% inhibition in the control clone versus 60% inhibition in the RunX2 overexpressing clone). The



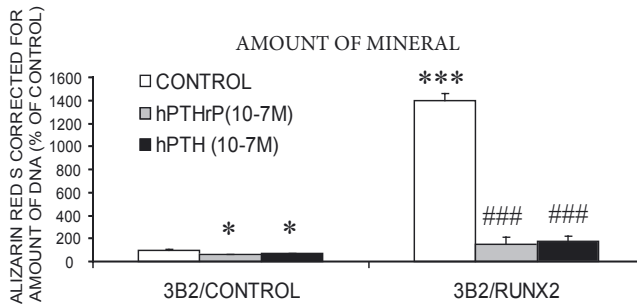
ALP-levels of the PTHrP- or PTH-treated RunX2 clones remained, however, higher than the highest level reached in the control clone. Much stronger inhibitory effects of hPTHrP (1-34) and hPTH (1-34) were observed on nodule formation and particularly matrix mineralization (fig. 5D and E) (36% inhibition in the control clone versus 89% inhibition in the RunX2 overexpressing clone).



5D



5E



**Fig. 5 Crosstalk between RunX2 and PTH/PTHrP signaling.**

Undifferentiated (day 4) or differentiated (day 11) KS483 cells were treated with  $10^{-7}$  M hPTHrP (1-34) for the indicated time periods. RunX2 (MASN) mRNA expression was measured with real time PCR (A). 3B2/control and 3B2/RunX2 clones were either left untreated or treated with  $10^{-7}$  M hPTHrP (1-34) or hPTH (1-34) for 72 hrs from day 4 onwards. RunX2 (MASN) mRNA expression was measured with real time PCR (upper part B). RunX2 protein expression was measured with Western Blot (lower part B). RunX2 and empty vector control clones were cultured until confluence and thereafter treated for 72 hrs. Basal ALP activity in the 3B2/RunX2 clones was significantly increased. Treatment with hPTHrP (1-34) or hPTH (1-34) dose-dependently decreased ALP activity in both control and RunX2 overexpressing clones (C). Continuous treatment with hPTHrP (1-34) and hPTH (1-34) for 18 days dose-dependently decreased the amount of ALP positive (D) and mineralized nodules (E) in empty vector control and RunX2 overexpressing clones. \*significant vs non-treated 3B2/control; # significant vs non-treated 3B2/RunX2 overexpressing clone.



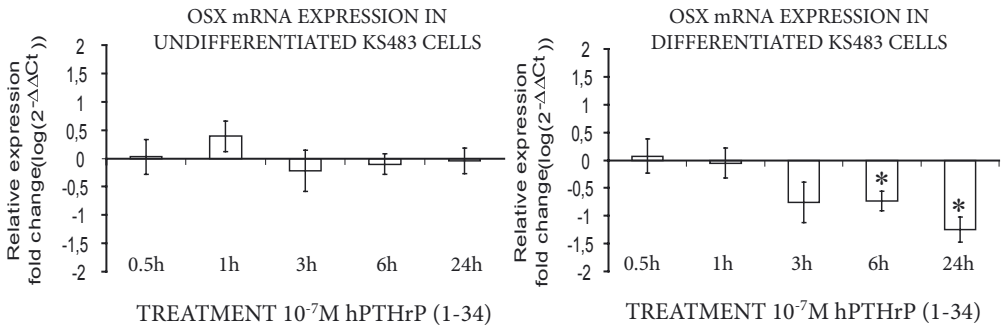
## Crosstalk between Osterix and PTH/PTHrP signaling

Osterix (*osx*) is another important transcription factor required for osteoblast differentiation acting downstream of RunX2<sup>(37)</sup>. We therefore tested whether bypassing RunX2 by forced overexpression of *osx* could reverse PTHrP and PTH mediated inhibition of osteoblast differentiation.

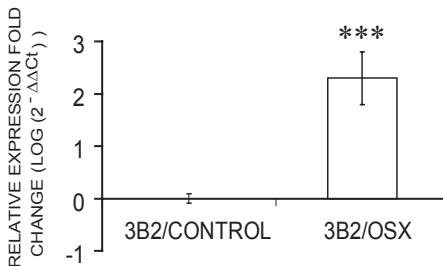
First, we investigated *osx* mRNA expression in undifferentiated and differentiated KS483 cells treated with hPTHrP (1-34) for the indicated time periods. As shown in figure 6A, hPTHrP (1-34) did not change mRNA expression of *osx* in undifferentiated cells. In contrast, *osx* mRNA expression was decreased from 3hrs treatment onwards by PTHrP treatment in differentiated cells. To test whether overexpression of *osx* could reverse the PTHrP inhibitory effects, we generated stable KS483 cell lines overexpressing *osx* (KSFrt 3B2/*osx*), which showed more than 100-fold overexpression of *osx* at the mRNA level (fig. 6B). Compared to the RunX2 overexpressing clone, *osx* overexpression had only a marginal effect on ALP activity. PTHrP and PTH inhibited ALP-activity to a similar extent in the control and *osx*-overexpressing clone (fig. 6C).

In long-term experiments, overexpression of *osx* resulted in a slight increased amount of ALP-positive nodules (fig. 6D). The most dramatic effect was observed on matrix mineralization (fig. 6D and E). Overexpression of *osx* could not overcome the inhibitory effects of hPTHrP (1-34) or hPTH (1-34), since they still caused a rapid decrease in the number and size of ALP positive nodules and potently inhibited matrix mineralization (respectively 6D and 6E).

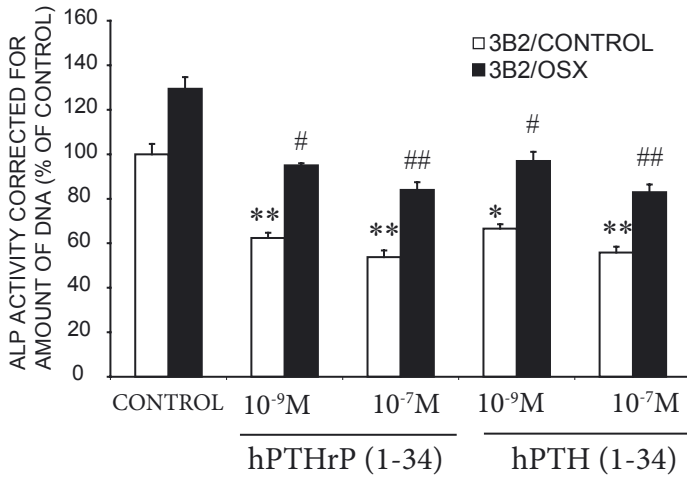
6A



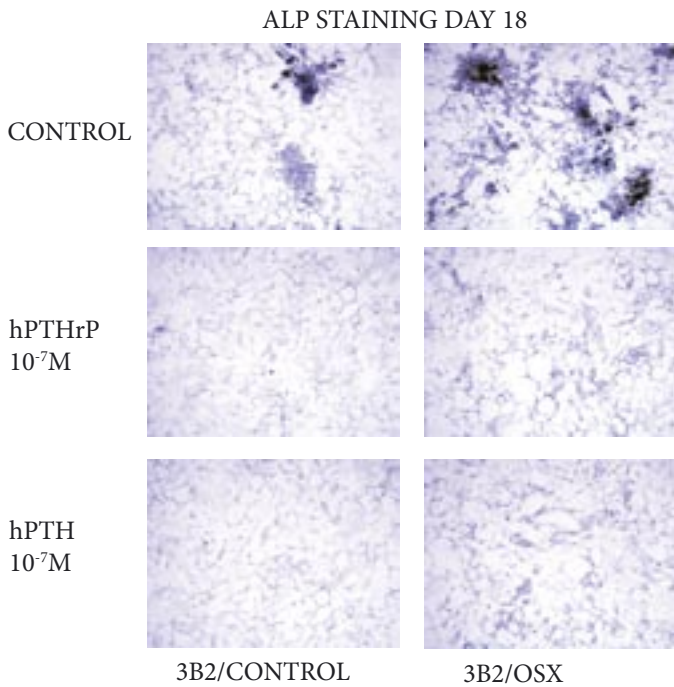
6B



6C

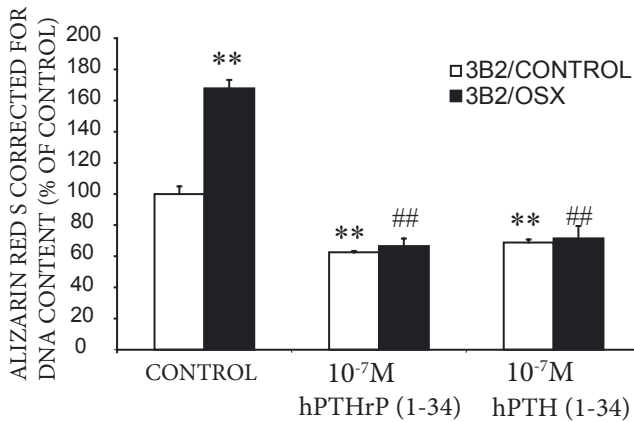


6D





6E



**Fig. 6 Crosstalk between Osterix and PTH/PTHrP signaling.**

Undifferentiated (day 4) or differentiated (day 11) KS483 cells were treated with  $10^{-7}$  M hPTHrP (1-34) for the indicated time periods. *Osx* mRNA expression was measured with real time PCR (A). *Osx* mRNA expression at day 4 of culture was significantly increased in the 3B2/*osx* clone (B).

*Osx* and empty vector control clones were cultured until confluence and thereafter treated for 72 hrs. Treatment with hPTHrP (1-34) decreased ALP activity in both 3B2/control and 3B2/*osx* clones. (C). The amount of mineralized nodules was significantly increased in 3B2/*osx* clones. Continuous treatment with  $10^{-7}$  M hPTHrP (1-34) for 18 days dose-dependently decreased the amount of ALP positive (D) and mineralized nodules (E) in both 3B2/control and 3B2/*Osx* clones. \*significant vs non-treated 3B2/control; # significant vs non-treated 3B2/*osx* overexpressing clone.

## Discussion

In this study we investigated the mechanism of action of PTHrP and PTH on osteoblast differentiation. Like in other osteoblastic cells, expression of the PTH1R and responsiveness to challenges with PTHrP and PTH increased during KS483 osteoblastic differentiation<sup>(53,54,55)</sup>. In KS483 cells, treatment with hPTH (1-34) or hPTHrP (1-34) resulted in inhibition of osteoblastic differentiation irrespectively of differentiation stage, exposure time and dosage. Inhibitory effects predominantly on matrix mineralization were also found in mouse bone marrow cultures. In these cells, ALP activity was inhibited in short-term cultures, while no differences were seen after long-term treatment. How this is regulated is presently unknown. Inhibition of differentiation and matrix differentiation by PTHrP and PTH is in line with the majority of previous *in vitro* studies<sup>(19,24,25)</sup>. How PTHrP or PTH inhibit osteoblastic differentiation is not known. They might influence the activity of the Hedgehog (Hh) or Bone Morphogenetic Protein (BMP) pathways to exert these effects, in a mechanism similar to that described in chondrocytes<sup>(56,57,58)</sup>. PTHrP or PTH might also influence the expression or activity of the osteoblast-specific transcription factors RunX2 and osterix (*osx*).

Since autocrine BMP signaling is important during all phases of osteoblast differentiation<sup>(32,59)</sup>, we proposed that PTHrP and PTH might inhibit osteoblast differentiation by interfering with the BMP signaling pathway. We demonstrated that in KS483 cells, BMP-induced osteoblast differentiation is inhibited by hPTHrP (1-34) or hPTH (1-34) and vice versa. This inhibitory effect was also found in MSC cultures, suggesting a

more widespread action of PTH and PTHrP at least *in vitro*. Our data are in line with data in chondrocytes, where BMP signaling stimulates chondrocyte maturation and can partially overcome the inhibitory effects of PTHrP on chondrocyte maturation<sup>(60)</sup>. In KS483 cells, further studies showed that hPTHrP (1-34)-mediated inhibition was downstream of early, Smad-dependent BMP signaling as well as downstream of BMP-induced p38 MAPK signaling, suggesting that the PTHrP-induced inhibition on BMP signaling is an indirect response.

PTHrP and PTH could also inhibit osteoblast differentiation by affecting the Hh pathway, another important pathway involved in osteoblastic differentiation<sup>(34,35)</sup>. Interactions between Hh and PTHrP have already been described in the growth plate, where they interact in a negative feedback loop<sup>(61)</sup>. In KS483 cells, Hh-induced ALP activity as well as nodule formation and mineralization could be inhibited by both hPTHrP (1-34) and hPTH (1-34) and vice versa. Inhibitory effects of PTHrP and PTH on hedgehog induced osteoblast differentiation were also found in MC3T3-E1 cells. In undifferentiated KS483 cells, PTHrP inhibited the expression of IHh mRNA. Moreover, mRNA expression of the Hh receptor Ptc1 and the signaling intermediates Gli1 and Gli3 was also decreased. Ptc1 and Gli1 are direct target genes of Hh<sup>(62)</sup> suggesting that PTHrP has an inhibitory effect on Hh signaling either directly or indirectly by downregulation of Hh expression. The mechanism by which Hh acts during osteoblast differentiation strongly resembles its action in CFK-2 chondrocytic cells where IHh induces chondrogenic differentiation, and PTHrP antagonizes Hh-mediated differentiation through a PKA-dependent mechanism<sup>(63)</sup>. Also in KS483 cells, PTHrP- and PTH-mediated inhibition of osteoblast differentiation was dependent on cAMP and PKA, since only PTHrP and PTH fragments with an intact N-terminus were able to elicit an effect.

PTHrP and PTH might also affect osteoblast differentiation by repressing the expression or activity of RunX2, an important transcription factor involved in osteoblast differentiation and a downstream target of Hh and BMP. The interactions between RunX2 and PTHrP and PTH signaling are complex. Effects of PTHrP and PTH signaling on RunX2 can occur at the level of mRNA transcription and protein expression<sup>(64,65)</sup>. In addition, PTHrP and PTH could regulate RunX2 activity by posttranslational modifications<sup>(66,67)</sup>. Indeed PTH can phosphorylate RunX2 in a PKA and MAPK dependent manner. These events are, however, associated with activation of the transactivation functions of RunX2. It seems, therefore, unlikely that these actions are involved in the inhibitory actions of PTHrP and PTH<sup>(66,68)</sup>. We demonstrated that PTHrP had no direct effect on transcription of RunX2, since treatment with PTHrP for 0.5, 1, 3, or 24 hours had no significant effect on its mRNA expression. In contrast, treatment of undifferentiated cells with PTHrP and PTH for 72 hrs prevented the increase in RunX2 mRNA and protein expression normally seen in osteoblast differentiation. This suggested that transcriptional regulation of RunX2, most likely indirect, plays an important role in the inhibition of osteoblast differentiation. Remarkably, overexpression of RunX2 driven by the EF1 $\alpha$ -promoter, which was relatively insensitive to regulation by PTHrP or PTH, was not able to reverse the inhibition. In fact, similar magnitudes of inhibition were found in the control clone and the RunX2-overexpressing clone using the early marker ALP as read out. This might be explained by a small decrease in RunX2 mRNA and protein expression compared to the untreated clone. However, the inhibition of matrix mineralization in the RunX2 clone was much stronger in comparison with the control



clone (36% versus 89%). This suggests that also other mechanisms may be involved in the inhibitory effects of PTHrP and PTH, such as posttranslational modification of RunX2, mechanisms downstream of RunX2 or RunX2-independent mechanisms. Since we did not observe a dissociation between RunX2 mRNA and protein expression, it seems unlikely that proteasomal degradation plays a major role in the mechanism of PTHrP- and PTH-mediated inhibition in KS483 cells<sup>(65)</sup>. PTHrP and PTH could also regulate the expression of other genes involved in matrix mineralization acting downstream of RunX2, such as *osx*. In KS483 cells, the mRNA expression of *osx* did not change during differentiation, in contrast with its expression pattern in fetal rat calvarial cells, which increased<sup>(69)</sup>. Indeed, in differentiated KS483 cells at the beginning of matrix mineralization, *osx* behaved like an intermediate early response gene of PTHrP. Downregulation of *osx* mRNA expression was already found after 6 hrs. Remarkably, this downregulation was not observed in undifferentiated cells. This could be due to the fact that in undifferentiated KS483 cells, low levels of the PTH1R are found and little cAMP response after PTH and PTHrP treatment. This may suggest that the genetic response to PTHrP and PTH changes with the differentiation stage of the osteoblast. This issue requires further study for example by comparing the genomic response to PTHrP and PTH in osteoblasts in distinct differentiation stages.

Overexpression of *osx* in KS483 cells predominantly stimulated late-stage osteoblast differentiation as assessed by matrix mineralization, while early osteoblast differentiation using ALP activity as read out was only borderline increased. This is in line with previous literature<sup>(37,70)</sup>. Forced overexpression of *osx* could not rescue the PTHrP-induced inhibition of ALP-activity in line with the absence of an effect of *osx* on early osteoblast differentiation. In addition, *osx* overexpression could not reverse the inhibition of matrix mineralization caused by PTHrP or PTH treatment. These data suggest that various mechanisms probably acting simultaneously or in parallel are responsible for the inhibition of matrix mineralization by PTHrP and PTH at least *in vitro*. Furthermore, we cannot exclude that PTHrP and PTH repress *osx* not only at the mRNA level but also at the posttranslational level by inhibiting its transactivation functions. The inhibitory effects of PTHrP and PTH on matrix mineralization, in which inhibition of *osx* expression may play a role, might be a relevant finding with respect to the anabolic actions of PTHrP and PTH on bone. The anabolic treatment regimes induce new bone which is relatively undermineralized<sup>(71)</sup>. Noteworthy in this respect is our observation that PTHrP and PTH potently inhibited matrix mineralization in long-term mouse bone marrow cultures without affecting ALP-activity, indicating that this action is more widely conserved and not restricted to KS483 cells.

Summarizing, we conclude that multiple mechanisms are involved in the inhibitory actions of PTHrP and PTH on osteoblast differentiation and matrix mineralization in KS483 cells. PTHrP and PTH inhibit BMP- and Hh-induced osteogenesis downstream of early BMP-signaling and by downregulation of components of the hedgehog signaling cascade. Inhibitory effects on early osteoblast differentiation may be explained by preventing the upregulation of RunX2, which is a downstream target of BMP and Hh-induced osteogenesis, by PTHrP and PTH in an indirect response. However, PTHrP and PTH still inhibit differentiation, and particularly matrix mineralization, of cells overexpressing RunX2. This could be at least partly explained by the capacity of PTHrP and PTH to downregulate *osx* expression at the beginning of matrix mineralization.



Like RunX2-overexpression, also *osx* overexpression could not relieve the inhibitory effects of PTHrP or PTH on matrix mineralization. These data indicate that besides transcriptional repression of RunX2 and *osx* also other mechanisms in parallel with or downstream of RunX2 and *osx* are involved in the inhibition of osteoblast differentiation and matrix mineralization by PTHrP and PTH.

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